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An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins

(oncogenes/DNA binding/dominant negative/regulation)

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ABSTRACT **Two forms of FosB transcript and their products can be identified in mouse NIH 3T3 cells following serum induction. The larger RNA codes for a 338-amino acid protein, whereas the smaller RNA results from the removal of an additional 140 nucleotides from FosB mRNA by alternative splicing. This alternative splicing event places a stop codon following the "leucine zipper" region and results in a shorter protein (FosB2) of 237 amino acids that lacks 101 amino acids at the carboxyl terminus. FosB2 is able to form heterodimers with c-Jun and bind to an AP-1 site but is not able to activate the transcription of promoters containing AP-1 sites. Furthermore, FosB2 can not only suppress the transcriptional activation by c-Fos and c-Jun of promoters containing an AP-1 site but also interferes with the transforming potential of viral and cellular Fos proteins. We propose that FosB2 protein functions as a trans-negative regulator.**

The immediate response of the cell to external stimulus results in the induction of a select set of genes, many of which are nuclear protooncogenes. Prominent among the products of these early response genes are Fos and Jun, which serve as a paradigm for cooperation between oncoproteins to activate transcription of specific promoters (1–4). The Fos and Jun proteins are members of the "leucine zipper" family and bind to AP-1 sites as heterodimers (5–7). Fos by itself is unable to form homodimers or bind to AP-1 sites (8, 9). In contrast, Jun can form homodimers but its subsequent binding to AP-1 site is very weak (10–13). Thus to achieve maximal levels of transcriptional transactivation, cooperation between the two nuclear oncoproteins is obligatory (4). There are at least four Fos-related genes referred to as c-*fos*, *fosB*, *fra1*, and *fra2*, all of which are inducible with a wide variety of agents (14–17). Similarly, there are three Jun-related genes, c-*jun*, *junB*, and *junD* (18–20). All of the Fos proteins can associate with Jun proteins to form heterodimers and bind to AP-1 sites with similar affinities (10, 20, 21). It is, therefore, enigmatic as to why the cell needs multiple forms of Fos- and Jun-related genes. One possibility is that these genes are differentially expressed during growth, differentiation, and development, although, in most cases studied so far, the expression of *fos* and *jun* genes appears to be coordinately regulated (4). Alternatively, it is possible that multiple forms of Fos and Jun proteins provide regulatory mechanisms. Indeed, c-Fos protein can negatively regulate the transcription of its own promoter but cooperates with Jun to augment the expression of c-*jun* gene (22–26). On the other hand, c-Jun protein positively regulates its own promoter but transcription of the c-*jun* gene is suppressed by its sibling, the JunB protein (26, 27).

During our investigation of the role of Fos-related proteins during cell proliferation, we found two distinct mRNA species encoding FosB proteins. Here we report that the larger mRNA coding for the previously described 338-amino acid FosB protein (15) contains a 140-base-pair (bp) intron that has an open reading frame. The shorter 237-amino acid FosB2 protein is the product of an alternatively spliced mRNA species, resulting in an in-frame stop codon. FosB and FosB2 proteins contain leucine zipper and basic region domains and bind to AP-1 sites. However, FosB2 interferes with the transcriptional transactivation and transformation potential of c-Fos and FosB, suggesting a role as a trans-negative regulator.

MATERIALS AND METHODS

FosB2 cDNA Cloning. Partial FosB2 cDNA that contains the entire coding sequence was obtained during cloning of the FosB cDNA by polymerase chain reaction (PCR) (28). Primers corresponding to nucleotides 1284–1301 and 2212–2227 of the published sequence (15) were used to clone FosB cDNAs using the PCR. Multiple independent clones were picked and analyzed by nucleotide sequencing.

Plasmid DNA Construction and RNase Protection Analysis. Plasmid SK⁻FosB2 contains the entire FosB coding sequence except the intron-derived exon (see text) in the vector SK⁻. Plasmid SK⁻FosB contains the entire FosB coding sequence. pBKFosB and pBKFosB2 are expression constructs of FosB and FosB2 in the Finkel–Biskis–Jinkins long-terminal-repeat-driven expression vector pBKPL. The analogous c-*fos* expression plasmid pBK28 and the c-*jun* expression plasmid pSV-c-jun have been described (20, 29). RNase protection analysis and Northern blot analysis were performed as described (20).

Protein Analysis by Immunoprecipitation. NIH 3T3 cells were starved with 0.5% bovine calf serum for 2 days and then stimulated with 10% dialyzed calf serum in the presence of methionine- and cysteine-free medium. Forty minutes before harvest, cells were labeled with [^{35}S]methionine (200 μCi/ml; 1 Ci = 37 GBq) and cell lysates were prepared as described (30). Lysates containing equal amounts of protein were used for immunoprecipitation with anti-FosB specific antibody 5108-1B (against FosB peptide residues from 80 to 97). Details of the preparation of FosB antibody will be published elsewhere.

***In Vitro* Transcription and Translation.** T7-c-Fos, pGEM-c-Jun, SK⁻FosB, and SK⁻FosB2 were linearized with *Eco*RI and transcribed with T7 polymerase or T3 polymerase. Each RNA was translated with micrococcal nuclease-treated, methionine-free rabbit reticulocyte lysate as described by the supplier (Promega). For gel shift assays, equal molar amounts of unlabeled proteins were combined and incubated with [γ-^{32}P]ATP-labeled PMA-responsive element/AP-1 (PMA = phorbol 12-myristate 13-acetate) oligonucleotide at room

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Abbreviations: CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.

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temperature; the binding complex formation was analyzed as described (7, 31).

DNA Transfection and Reporter Gene Assays. Embryonic carcinoma F9 cells were plated in 10-cm dishes at a density of 5×10^5 cells per plate 24 hr before transfection. Transfection protocol was as described (32), and β-galactosidase activity was assayed by the *o*-nitrophenyl β-galactoside reaction (33). For chloramphenicol acetyltransferase (CAT) assays, cell extracts containing equal β-galactosidase activity were incubated with [^{14}C]chloramphenicol, and the reaction products were analyzed on TLC plates as described (34).

RESULTS

Characterization of FosB2 cDNA. During the process of molecular cloning of FosB cDNA by PCR using oligonucleotides based on the published FosB sequence (15), we obtained DNA fragments of ≈0.9–1.0 kilobase pair (kbp). These cDNA fragments were subcloned and when their nucleotide sequence was determined, it became apparent that many of them were missing 140 bp compared to previously described FosB cDNAs. More importantly, the open reading frame of the shorter cDNA could encode a protein of only 237 amino acids as compared to the 338-amino acid protein encoded by the FosB cDNA. Fig. 1*A* schematizes the molecular structure of the FosB cDNA and the shorter cDNA termed FosB2. The nucleotide sequence and deduced amino acid sequence of the first 237 amino acids of the two proteins are identical (15). Examination of the sequences of FosB cDNA and FosB2 cDNA revealed that the FosB2 protein will terminate immediately following the 140-bp deletion. Thus the remaining sequence in the 3′ region of FosB2 is identical to FosB but cannot be translated in this frame. The FosB2 protein, however, does contain the leucine zipper domain (amino acids 183–211) and the basic region (amino acids 157–182), which is well conserved among all members of the *fos* family (16).

To address the question of the nature of the 140-bp insert in FosB, we determined the nucleotide sequence of a corresponding FosB genomic DNA fragment. A comparison of the sequences of FosB genomic DNA, FosB cDNA, and FosB2 cDNA revealed that the 140-bp insert has all of the hallmarks of a bonafide type II intron (Fig. 1*B*). In addition to the consensus splice donor (AG↓GTGAGA) and acceptor (CAG) sites, there are potential branch points 23 and 28 nucleotides from the AG and a pyrimidine-rich stretch between branch point and splice site. Because the 140-bp intron is deleted in FosB2, the reading frame shifts by −1, creating the stop codon TGA. Therefore FosB2, the product of spliced FosB mRNA, is only 237 amino acids long.

FosB2 and FosB Transcription Are Coordinately Regulated Following Serum Induction. Southern blotting analysis of genomic DNA suggested the presence of a single *fosB* gene (data not shown). We also prepared RNA from cells induced with serum and hybridized to FosB2 probe. Fig. 2*A* shows that by 60 min of serum induction, maximal levels of a 5.0-kb species of RNA can be detected (lane 2). Because the alternative spliced form of FosB RNA will be shorter by only 140 bp, the analysis performed here is not able to distinguish between the two forms of RNA. To confirm the existence of alternatively spliced forms of FosB mRNAs, we performed RNase protection analysis using probes generated from either FosB2 or FosB cDNA (Fig. 2*B*). The expected size of the protected fragments is indicated in Fig. 2*C*. The probes in Fig. 2*B* (lanes 1–6) are derived from FosB2 cDNA. Upon annealing with full-length FosB mRNA and subsequent RNase digestion, a 166-nucleotide fragment diagnostic for FosB RNA is expected. The results indicate that (*i*) bands of the expected sizes corresponding to FosB and FosB2 RNA can be identified, (*ii*) FosB and FosB2 expression is stimulated by serum (Fig. 2*B*, lane 4), and (*iii*) induction of FosB and FosB2 mRNA by serum can be superinduced with cycloheximide (Fig. 2*B*, lane 6). No induction of FosB or FosB2 mRNA can be detected when the cells are treated with PMA (Fig. 2*B*, lane 5). FosB is thus unique among early-response genes in that it is inducible with serum but not by PMA. For precise quantitation of the abundance of two species of RNAs we performed RNase protection analysis using probes generated from FosB cDNA.

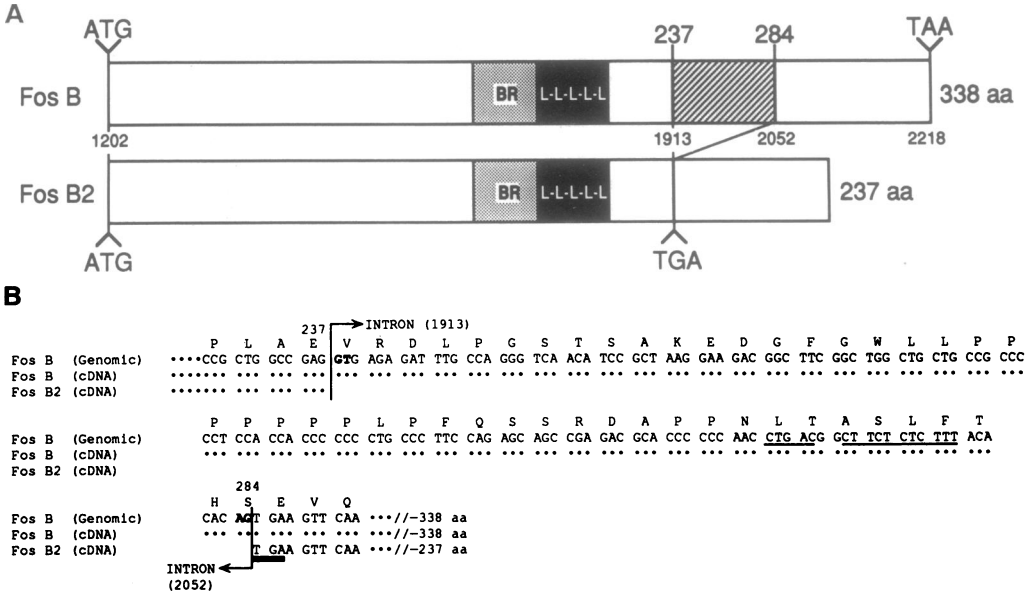

FIG. 1. Structure of FosB2 coding sequence. (*A*) Schematic representation: Alignment of structural features of FosB2 coding region with that of FosB cDNA. The entire coding sequence from nucleotide 1202 to 2218 (based on numbering system in ref. 15) including the intron-derived exon from nucleotide 1913 to 2052 (the hatched box) is shown. The stop codon (TGA) in the FosB2 reading frame is indicated. The BR box represents the basic region, and the L-L-L-L-L box indicates the leucine zipper. aa, amino acids. (*B*) Sequence of the alternative spliced intron of *fosB* gene. The nucleotide sequence of mouse genomic DNA surrounding the alternative intron of *fosB* gene is shown in the top line. FosB and FosB2 sequences are aligned and presented in the middle line and bottom line, respectively. The conserved splicing donor (GT) and acceptor (AG) are shown in boldface type. The consensus branch point sequence (CTGAC) and a pyrimidine stretch that is involved in lariat formation are underlined. The stop codon (TGA) of FosB2 cDNA is also underlined; . . . . indicates complete identity.

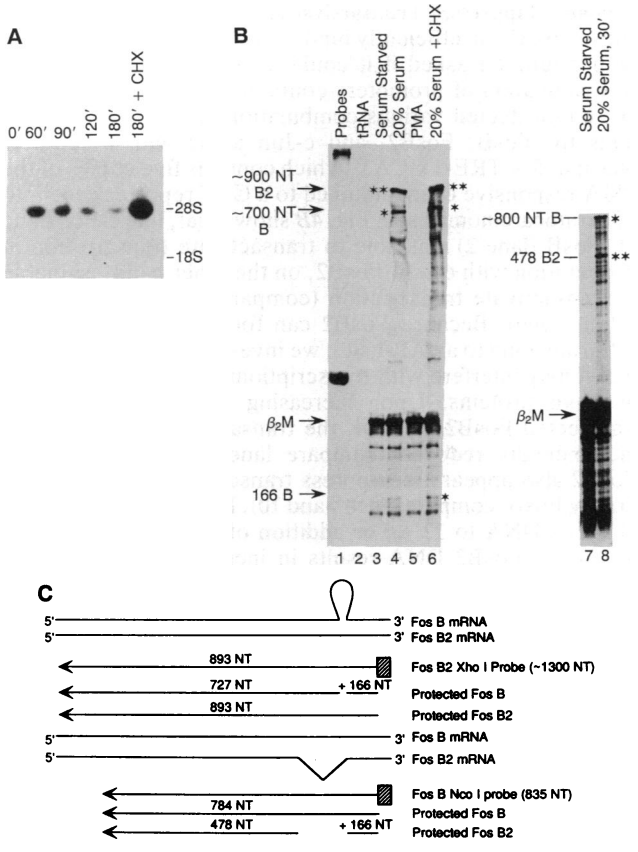

FIG. 2. Alternative spliced mRNA transcripts of FosB gene. (*A*) Serum induction of FosB mRNA. Five micrograms of cytoplasmic RNA was subjected to RNA blot analysis. The FosB2 coding region of cDNA was used as hybridization probe. CHX, cycloheximide. (*B*) Coordinated regulation of FosB and FosB2 mRNA by serum in NIH 3T3 fibroblasts. Lane 1, uniformly [^{32}P]UTP-labeled complementary RNA probes were synthesized [FosB2 *Xho* I probe and β_2-microglobulin (β_2M) probes were included in this experiment]; lane 2, 20 μg of yeast tRNA; lane 3, 20 μg of cytoplasmic RNA from serum-starved cells; lane 4, 20 μg of cytoplasmic RNA of 20% serum-stimulated cells (for 30 min); lane 5, PMA-treated cells; lane 6, 20 μg of RNA of serum- and cycloheximide-treated cells (for 60 min); lanes 7 (serum-starved) and 8 (serum-stimulated 30 min), FosB *Nco* I fragment was used as probe. NT, nucleotides. (*C*) Schematic presentation of the structures of FosB- and FosB2-specific protected fragments. The loop indicates the intron-derived exon. The FosB2 and FosB riboprobes are depicted and the extra 51 nucleotides derived from the T7 transcribed vector sequence are as shown in the hatched boxes. The sizes of each probe and protected fragment are indicated.

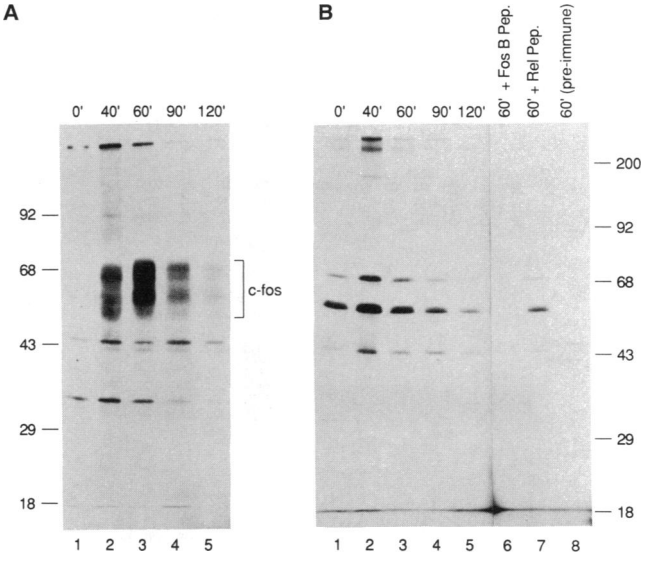

FIG. 3. Analysis of FosB and FosB2 proteins. (*A*) Expression of c-Fos protein during serum stimulation. (*B*) Expression of FosB-related proteins during serum stimulation. The antibody was preincubated with FosB peptide (amino acids 80–97) or Rel peptide (amino acids 152–176) before the immunoprecipitation (lanes 6 and 7, respectively). Preimmune serum was used in lane 8. In *A* and *B* the lysates were boiled before immunoprecipitation; therefore no Jun-related proteins can be identified. Molecular masses are given in kDa.

Fig. 2*B* (lane 8) shows that in cells stimulated with serum, appropriate length FosB- and FosB2-specific fragments can be identified. From densitometric scanning of the band intensity, length of fragments, and uracil content of fragments, it appears that FosB2 is slightly more abundant (FosB/FosB2 = 0.83/1) than FosB mRNA.

Synthesis of FosB and FosB2 Protein Is Inducible. We next looked for protein products of FosB mRNA transcripts by immunoprecipitation of [^{35}S]methionine-labeled cultures using a FosB-specific antiserum (5108-1B) directed against an 18-amino acid FosB peptide (residues 80–97) or a c-Fos monoclonal antibody, 18H6. Fig. 3*A* shows that several extensively phosphorylated forms of c-Fos protein can be immunoprecipitated with 18H6 antibody with maximal levels between 30 and 60 min followed by a marked decrease by 120 min. No Jun-related proteins are identified because the extracts were boiled, which disrupts the Fos–Jun complex prior to immunoprecipitation. In parallel experiments, 5108-1B antiserum immunoprecipitated three polypeptides of approximate molecular masses 65 kDa, 55 kDa, and 43 kDa after serum induction. These polypeptides could be identified in quiescent cells (lane 1), but the levels increased by 40 min (lane 2) and declined to basal levels by 90–120 min (lanes 4 and 5). When the antibody was preincubated with synthetic peptide against which the antiserum was raised, two of the polypeptides could not be detected (lane 6). On the other hand, the immunoprecipitation of the two polypeptides was not affected if the antiserum was preincubated with a nonspecific peptide (amino acids 152–176 of murine c-Rel) originating from c-Rel protein (lane 7). Neither the 65- nor 55-kDa polypeptide could be observed with the preimmune serum (lane 8). Since we do not have an antiserum that distinguishes between FosB and FosB2, we cannot distinguish which of the two polypeptides is the product of FosB or FosB2 RNA. However, both polypeptides, as well as *in vitro* translated FosB protein, show common peptides following digestion with V8 protease (data not shown). From the molecular masses of the *in vitro* translated FosB and FosB2 RNA transcripts and the possible posttranslational modifications we would assign the 65-kDa polypeptide as FosB and the 55-kDa protein as FosB2. The lower band is nonspecifically precipitated by protein A-Sepharose (lane 8) and could not be blocked by FosB peptide (lane 6). It is likely to be actin, which is inducible with serum and migrates at a position of about 43 kDa (also see this band in Fig. 3*A*, lane 1, with Fos monoclonal antibodies).

FosB2 Binds to an AP-1 Site. A hallmark of the members of the *fos* gene family is that their products form heterodimers with members of the *jun* family and bind to AP-1 sites. We therefore asked if FosB2 alone or in association with c-Jun binds to an AP-1 site. Fig. 4*A* shows that, like FosB (lanes 7 and 8), *in vitro* translated FosB2 and c-Jun can efficiently bind to an AP-1 site (lane 9). Competition with excess unlabeled oligonucleotide containing the cognate AP-1 site abolishes binding (lane 10). Little or no binding is observed with either FosB2, FosB, c-Fos, or c-Jun alone (lanes 3–6). To confirm that DNA binding by the FosB2–c-Jun complex

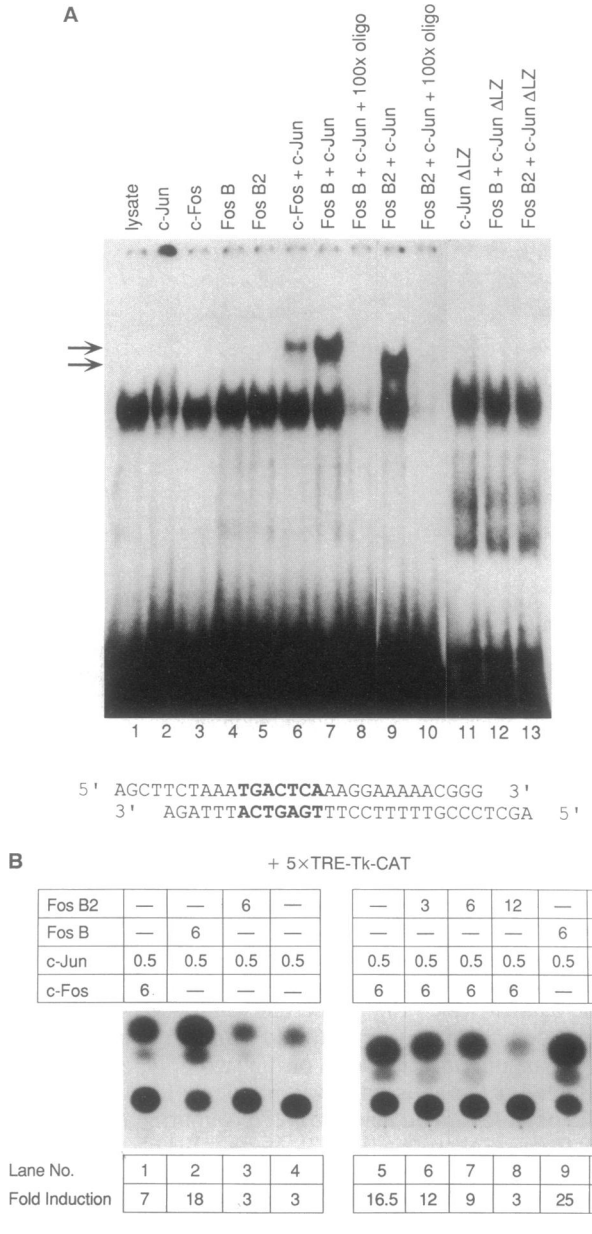

FIG. 4. DNA binding and transactivation by FosB2. (*A*) FosB2–c-Jun heterodimer binds to an AP-1 site. Equal amounts of unlabeled *in vitro* translated proteins were mixed with 3×10^4 cpm of [γ-^{32}P]ATP-labeled AP-1 oligonucleotide (sequence is shown, with the core sequence of the AP-1 site in boldface type) at room temperature for 15 min. The reaction products were analyzed on a native 4% acrylamide gel. Positions of the FosB–c-Jun–AP-1 complex and FosB2–c-Jun–AP-1 complex are indicated by arrows. (*B*) Suppression of transactivation by FosB2. Two micrograms of the reporter construct 5 × TRE-Tk-CAT and 2 μg of pBAG (a β-galactosidase expression plasmid that served as an internal control of transfection efficiency) were cotransfected into F9 cells with various combinations of expression plasmids. The amount of each expression plasmid transfected (in μg) is shown in the top panel. Forty-eight hours after transfection CAT activity was measured. All CAT activity assays were standardized with β-galactosidase activity. Fold induction was standardized with the control reaction (no expression plasmid) and three independent experiments were performed. The expression plasmids pBKFosB, pBKFosB2, pBK28, and pSV-c-jun are described in the text.

is dependent on heterodimer formation by means of the leucine zipper domain, we cotranslated *in vitro* transcribed RNAs encoding FosB2 and a c-Jun mutant containing a deletion of the leucine zipper (13). No binding to the AP-1 site (lanes 11–13) could be detected.

FosB2 Suppresses Transactivation by c-Fos and FosB. Because FosB2 can efficiently bind to an AP-1 site in association with c-Jun, we asked if it could also cause transcriptional transactivation of promoters containing AP-1 binding sites. We cotransfected various combinations of expression plasmids for FosB, FosB2, and c-Jun along with a reporter plasmid, 5 × TRE-Tk-CAT, which contains five copies of the PMA-responsive element linked to a CAT reporter gene into F9 teratocarcinoma cells. Fig. 4*B* shows that, like c-Fos (lane 1), FosB (lane 2) was able to transactivate transcription in cooperation with c-Jun. FosB2, on the other hand, is unable to transactivate transcription (compare lanes 3 and 4 with c-Jun alone). Because FosB2 can form heterodimers with c-Jun and bind to an AP-1 site, we investigated the possibility that it may interfere with transcriptional activation by c-Fos or FosB proteins. Upon increasing the concentration of transfected FosB2 plasmid, the transactivation by c-Fos is incrementally reduced (compare lane 5 with lanes 6–8). FosB2 also appears to suppress transcriptional transactivation by FosB (compare lanes 9 and 10). Increasing the amount of c-Fos DNA to 12 μg or addition of 6 μg of FosB DNA instead of FosB2 DNA results in increased CAT activity, indicating that reduced CAT activity upon addition of FosB2 plasmid is not due to adventitious events like squelching (data not shown). Furthermore, immunostaining of the transfected cells with c-Fos monoclonal antibody (18H6) shows that c-Fos protein is synthesized (data not shown). Finally, FosB2 mutants in the leucine zipper domain are unable to suppress transactivation by c-Fos and c-Jun (data not shown). Thus the results shown here suggest that FosB2 can interfere with transcriptional transactivation by c-Fos and FosB proteins.

FosB2 Suppresses Transformation by Fos Proteins. The ability of FosB2 to suppress transcriptional transactivation by c-Fos and FosB prompted us to ask the question if it can also interfere with their transforming potential. Table 1 shows that increasing amounts of FosB2 plasmid decreased the transforming potential of v-Fos, c-Fos, and FosB proteins. In agreement with our previous results, v-Fos is a more potent transforming agent than its cellular cognate, c-Fos (35). Surprisingly, however, FosB appears to have a transforming potential equivalent to that of the v-*fos* gene. These results also emphasize a link between transactivation potential and cellular transformation by Fos proteins.

DISCUSSION

In the past few years it has become clear that alternative splicing is an important mechanism for the regulation of

Table 1. Suppression of transformation by FosB2

Transforming DNA	FosB2, μg	Number of foci		Transformation efficiency (average)
		Exp. 1	Exp. 2	
v-Fos	0	130, 108	55, 67	100
	2	58, 75	30, 48	60
	16	36, 38	9, 15	25
c-Fos	0	43, 31	21, 26	100
	2	16, 8	5, 3	17
	16	2, 8	5, 2	15
FosB	0	163, 175	48, 61	100
	2	102, 82	43, 25	51
	16	38, 36	28, 17	31
None	16	0, 0	0, 0	

208F cells were transfected with 2 μg of transforming plasmid DNA (v-Fos, c-Fos, or FosB) along with different amounts of a FosB2 expression plasmid; the total amount of DNA used in each transfection was kept constant by varying the amount of carrier DNA. Focus assays were performed as described (35). Each transfection was plated in duplicate. Foci were counted 12 days after transfection for v-Fos-transfected cells and 17 days after transfection for c-Fos- and FosB-transfected cells.

synthesis of numerous biologically important proteins (36). The simplest form of such regulation results from tissue- or temporal-specific splicing events that lead to the synthesis of either productive (protein-coding) or nonproductive (nonprotein-coding) RNAs. Alternative splicing also manifests biological importance by including or excluding particular coding sequences within the mRNAs. Thus the biological function of a single gene can be expanded by the splicing choice. Several oncogenes and protooncogenes are known to encode more than one product by generating alternatively spliced mRNAs, including the simian virus 40 large tumor antigen gene, c-*src*, c-Ha-*ras*, c-*abl*, and c-*myb* (36–41). The alternate splicing of c-*myb* is particularly instructive; an alternatively spliced c-Myb mRNA that encodes a truncated version of $p75^{c\text{-}myb}$ (*mbm2*), which includes the DNA binding region and nuclear localization signal present in c-Myb protein but lacks regulatory regions required for transcriptional activation, has been reported (41). The truncated protein, *mbm2*, interferes with the function of c-Myb during differentiation of mouse erythroid leukemia cells (41). Our data suggest that FosB2/FosB interactions are functionally analogous to the c-Myb/*mbm2*. FosB2 associates with c-Jun to form heterodimers, which bind to AP-1 sites as heterodimers but which lack transcriptional activation properties.

The *fosB* gene belongs to a multimember family, all of which exhibit the following characteristics: (*i*) transient inducibility with a wide variety of agents and (*ii*) short half-life of the products, which are invariably posttranslationally modified, usually by phosphorylation. Fos proteins are active only through heterodimer formation with members of the *jun* family; the resulting heterodimer activates transcription of genes containing AP-1 binding sites (1, 3). The inability of FosB2 to activate transcription in the presence or absence of Jun protein suggests that the transactivating domain of FosB lies at its carboxyl terminus. Interestingly, inspection of the 47-amino acid region of FosB encoded by the 140-bp intron (Fig. 1) reveals the presence of a proline-rich region previously proposed to constitute a transactivation domain (42). It will be of interest to see if fusion of this 47-amino acid proline-rich domain to FosB2 would convert the latter into an activator of transcription.

Identification of FosB2 introduces a new element of regulation into the complex interactions between the various members of the *fos* and *jun* family. Could FosB2 act as an additional regulator by titrating the amount of the Jun proteins available to form complexes with Fos proteins to activate transcription? In preliminary experiments it is clear that FosB2 can form heterodimers with not only c-Jun but also JunB and JunD (J.Y., unpublished data). Furthermore, FosB2 complexed with JunB and JunD can also bind to AP-1 sites (J.Y., unpublished data). Since *fos* and *jun* genes are often transcribed in response to external signals, perhaps the generation of truncated FosB2 will functionally inactivate the Fos–Jun complex. The challenge is to identify specific cell types in which the alternate splice choice is regulated to alter the ratio of FosB to FosB2.

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